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Effects of Cellular, Chemical, and Pharmacological Chaperones on the Rescue of a Trafficking-defective Mutant of the ATP-binding Cassette Transporter Proteins ABCB1/ABCB4*

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Background: Mutations of ABCB4, a transporter highly homologous to ABCB1, cause severe liver disease.
Results: The I541F mutation induces misfolding and intracellular retention that is rescued by the ABCB1-competitive substrate cyclosporin A but not by modulating the chaperones calnexin or Hsp/Hsc70.
Conclusion: Pharmacological chaperones are potential therapeutic tools for ABCB4 misfolded mutants.
Significance: This opens perspectives to treat ABCB4-linked genetic diseases.

The ATP-binding cassette transporter ABCB4 is a phosphatidylcholine translocator specifically expressed at the bile canalicular membrane in hepatocytes, highly homologous to the multidrug transporter ABCB1. Variations in the *ABCB4* gene sequence cause progressive familial intrahepatic cholestasis type 3. We have shown previously that the I541F mutation, when reproduced either in ABCB1 or in ABCB4, led to retention in the endoplasmic reticulum (ER)/Golgi. Here, Madin-Darby canine kidney cells expressing ABCB1-GFP were used as a model to investigate this mutant. We show that ABCB1-I541F is not properly folded and is more susceptible to *in situ* protease degradation. It colocalizes and coprecipitates with the ER chaperone calnexin and coprecipitates with the cytosolic chaperone Hsc/Hsp70. Silencing of calnexin or overexpression of Hsp70 have no effect on maturation of the mutant. We also tested potential rescue by chemical and pharmacological chaperones. Thapsigargin and sodium 4-phenyl butyrate were inefficient. Glycerol improved maturation and exit of the mutant from the ER. Cyclosporin A, a competitive substrate for ABCB1, restored maturation, plasma membrane expression, and activity of ABCB1-I541F. Cyclosporin A also improved maturation of ABCB4-I541F in Madin-Darby canine kidney cells. In $HepG_2$ cells transfected with ABCB4-I541F cDNA, cyclosporin A allowed a significant amount of the mutant protein to reach the membrane of bile canaliculi. These results show that the best strategy to rescue conformation-defective ABCB4 mutants is provided by pharmacological chaperones that specifically target the protein. They identify cyclosporin A as a potential novel therapeutic tool for progressive familial intrahepatic cholestasis type 3 patients.

Most proteins need to fold into a correct three-dimensional structure to be functional. Failure to fold into the native shape usually produces inactive proteins that are degraded. Folding of secretory and transmembrane proteins is assisted by specialized chaperones associated with the endoplasmic reticulum (ER)[2] (1, 2). The role of these chaperones is to prevent aggregation of not yet folded polypeptides and to exert quality control, thus ensuring that only correctly folded molecules are transported to their final destination (3, 4). Proteins unable to reach the native state are recognized as misfolded and targeted to the ER-associated degradation system (5, 6). In this case, they are transported back to the cytosol and eventually subjected to proteolysis.

Many inherited diseases are associated with defective protein folding (7). In most cases, the folding defect is caused by single point missense mutations that prevent the protein to reach its correct tertiary structure. Consequently, the defective protein is retained in the ER in interaction with cellular chaperones and is not transported to its final destination, which results in loss of function. However, the mutant protein might be fully functional if it could escape the quality control machinery. When processed at reduced temperature, several mutant proteins are able to reach a native conformation compatible with their exit from the ER and with their functionality (8–10). Therefore, a challenge is to find pharmacological means to rescue the proper targeting and functionality of misfolded proteins. The main strategies that are being developed are to modulate the activity of cellular chaperones involved in the folding and quality control of the protein of interest (11) or to use drugs that act as chemical or pharmacological chaperones (7, 12–15).

Multidrug-resistant proteins MDR1/ABCB1 and MDR3/ABCB4 are closely related ATP-binding cassette (ABC) transporters. They are composed of two moieties, each containing six membrane-spanning segments and a nucleotide-binding

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[2] The abbreviations used are: ER, endoplasmic reticulum; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane regulator; endoH, endo-β-*N*-acetylglucosaminidase H; 4-PB, 4-phenyl-butyrate.

domain. Although they share almost 80% identity at the amino acid level, their functions are quite distinct. ABCB1, the membrane drug transporter P-glycoprotein, is rather ubiquitous and extrudes a large variety of amphiphilic drugs. ABCB4 is mainly expressed at the bile canalicular membrane of hepatocytes, where it is specialized in biliary phosphatidylcholine excretion. Mutations in the *ABCB4* gene cause several biliary diseases, including progressive familial intrahepatic cholestasis type 3, a rare disease characterized by the early onset of persistent cholestasis that progresses toward cirrhosis and liver failure before adulthood (16). We have previously investigated effects of the I541F *ABCB4* mutation that has been identified in a progressive familial intrahepatic cholestasis type 3 patient. The mutation is located in the first nucleotide-binding domain, which is highly homologous between ABCB1 and ABCB4. This mutation had similar effect when reproduced in either ABCB1 or ABCB4, leading to intracellular trafficking defect and ER/Golgi retention (10). Nevertheless, intracellular traffic was restored when cells were grown at 27 °C, and the rescued ABCB1-I541F mutant was functional. The aim of this work was to test other means to provide functional rescue for the I541F mutation. We have taken the ABCB1-I541F mutant as a model to explore different strategies. ABCB1-I541F has the advantage that it can be expressed at detectable level as a GFP fusion protein and ABCB1 activity is easy to measure. We have determined the effect of modulating two ER chaperones (calnexin and Hsp/Hsc70) expression and analyzed the potential effect of chemical and pharmacological chaperones that have proven effective in rescuing the traffic of other folding-defective mutant proteins. We found that cyclosporin A, an ABCB1 substrate, induced the best rescue of the ABCB1-I541F mutant and also provides rescue of the ABCB4-I541F mutant.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit polyclonal anti-calnexin and anti-Hsc70 antibodies were purchased from Enzo Life Sciences (Villeurbanne, France). The mouse monoclonal anti-ABCB4 P3-II-26 antibody was from Alexis Biochemicals (San Diego, CA), the mouse monoclonal anti-Hsp70 from Abcam (Cambridge, UK), the mouse monoclonal anti-GFP from Roche (Meylan, France), the mouse monoclonal anti-GM130 from BD Biosciences France (Le Pont-de-Claix, France), and the goat polyclonal anti-actin from Santa Cruz Biotechnology (Heidelberg, Germany). Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (Montluçon, France); Alexa Fluor 488 secondary antibodies and culture media were from Invitrogen, and peroxidase-conjugated secondary antibodies were from Rockland Immunochemicals (Gilbertsville, PA). Endo-β-*N*-acetylglucosaminidase H and peptide *N*-glycosidase F were from Roche. Calcein-AM was from Anaspec (San Jose, CA). Protein G-Sepharose and the ECL-Plus detection kit were from GE Healthcare (Orsay, France). Thapsigargin, thermolysin and kallikrein were from Sigma-Aldrich Chimie (Lyon, France). Sodium 4-phenylbutyrate was from Biovision (Clinisciences, Montrouge, France). Cyclosporin A and verapamil were from Calbiochem (Merck Chemicals Ltd., Nottingham, UK). The siRNA-calnexin duplex was obtained from Eurogentec (Liège, Belgium) and control siRNA from Dharmacon Research (Lafayette, IL). The Hsp70 cDNA was a kind gift of L. R. Choo-Kang (Johns Hopkins University Medical School, Baltimore, MD).

Cell Culture, Transfection, and Drug Treatments—Madin-Darby canine kidney (MDCK) II cells and hepatocellular carcinoma, human $HepG_2$ cells were grown at 37 °C in Dulbecco's modified medium as reported (10). The generation of MDCK cells stably expressing GFP-ABCB1, GFP-ABCB1-I541F, ABCB4 and ABCBA-I541F has been previously described (10). Before the experiments, cells were treated overnight with 10 mM sodium butyrate to increase the cytomegalovirus promoter transcriptional activity. Transient transfections were performed in 6-well plates with 2 μg of plasmid per well, using either the Nucleofector II system (Lonza, Levallois-Perret, France) or the Turbofect reagent (Fermentas France, Villebon-sur-Yvette). Stock solutions of drugs were made at 1000× concentration in dimethyl sulfoxide (thapsigargin), ethanol (cyclosporin A), or PBS (4-phenylbutyrate), and stored at −20 °C. Control cells were incubated with vehicle alone.

Isolation of Microsomes and Limited Proteolytic Digestion—MDCK cells (2.5×10^6) were grown in P150 Petri dishes for 6 days. Cells were washed twice with cold PBS, scraped with a rubber policeman, and suspended in HS medium (10 mM HEPES, 0.25 M sucrose (pH 7.4)). Microsomes were isolated by nitrogen cavitation as described (17). The suspension was equilibrated with nitrogen at 1000 psi in a high pressure chamber (Parr Instrument Company, Moline, Il) for 15 min at 4 °C. The cell suspension was rapidly expanded against atmospheric pressure and centrifuged at 3000 rpm for 10 min at 4 °C to remove cell debris and nuclei. The supernatant was then centrifuged at 24,200 rpm for 1 h at 4 °C in a SW41 swinging bucket rotor. Pellets were resuspended in HS. Isolated microsomes were incubated with kallikrein or thermolysin at the indicated concentrations for 30 min at 4 °C in digestion buffer (10 mM HEPES, 0.15 M NaCl (pH 7.4) with 4 mM $CaCl_2$ in the case of thermolysin). Samples were immediately denatured in 2× Laemmli sample buffer for 10 min at 50 °C and electrophoresed on 4–12% SDS-polyacrylamide gels. Immunoblotting was performed using the mouse anti-GFP monoclonal antibody.

RNA Interference—Twenty-one-nucleotide duplexes corresponding to calnexin coding nucleotides aatgtggtggtgcctatgtga with symmetric thymidine overhangs were used (31). This sequence is entirely conserved in the human and canine genomes. MDCK cells were transfected with 100 nM siRNA duplex using the Turbofect transfection reagent. Cells were studied 72 h after transfection when silencing of calnexin was effective.

Immunofluorescence and Confocal Microscopy—MDCK cells were grown either on coverslips or on Transwell polycarbonate filter units (Costar Corp., Cambridge, MA). Cells expressing ABCB1 were fixed with 4% paraformaldehyde and subsequently permeabilized with 0.075% saponin. Cells expressing ABCB4 were fixed with methanol/acetone (4:1, v/v) at −20 °C. Incubations with primary and secondary antibodies were performed as described (10). Confocal imaging was acquired with a Leica TCS SP2 laser scanning spectral system attached to a DMR inverted microscope with a 63/1.4 immersion objective.

Digital images were analyzed using the on-line ScanWare software and processed with Image J and Photoshop softwares.

Immunoprecipitation, Deglycosylation, and Western Blot—Cells were lysed on ice for 30 min in 10 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 1% digitonin, 10 mM iodoacetamide, 2 mM phenyl-methyl-sulfonylfluoride (pH 7.3) in the presence of a protease inhibitor mixture (Roche). Lysates were centrifuged at 12,000 × *g* for 10 min to remove insoluble materials. Protein content was determined by Uptima bicinchoninic acid protein assay from Pierce (Interchim, Montluçon, France). Immunoprecipitation was performed overnight with 1 mg of lysate protein and 2 μg of the monoclonal anti-GFP preadsorbed onto protein G-Sepharose beads for 4 h at 4 °C. Endoglycosidase digestions were performed for 1 h at 37 °C after lysing the cells in 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 20 mM Tris-HCl buffer (pH 7.4) (with 0.5% SDS in the case of Endo H) using 5μU endo-β-*N*-acetylglucosaminidase H or 1 unit peptide *N*-glycosidase F. Samples were denatured in Laemmli sample buffer for 30 min at 37 °C and run on 6% SDS-polyacrylamide gels. Immunoblotting was performed using horseradish peroxidase-conjugated secondary antibodies. Development of peroxidase activity was performed with the ECL Plus detection kit. Bands on gels were scanned and quantified using ImageJ software.

Calcein Assay—The procedure was essentially performed as described (18). Cells were seeded at 70,000 cells/well in 96-well plates with clear bottoms (Greiner Bio-One, Les Ulis, France). The medium was changed 24 h after seeding, and the assay was performed 48 h later. Monolayers were washed three times with Leibowitz's medium. Calcein-AM was added at a final concentration of 5 μM and 0.1% dimethyl sulfoxide in 100 μl Leibowitz's medium. Maximum calcein fluorescence was measured in cells treated with 50 μM verapamil that was added 10 min before and during the assay. Plates were incubated for 30 min at 37 °C, and fluorescence was measured in a Tecan SpectraFluor cytofluorimeter (MTX Lab Systems, Vienna, VA) at 485 nm excitation and 530 nm emission.

Statistics—Student's *t* test was used for statistical comparisons.

RESULTS

The I541F Mutant Is More Susceptible to Protease Degradation—We previously showed that the I541F mutant was retained in the ER and that retention could be rescued by low temperature, suggesting that the mutant had a folding defect (10). Improperly folded molecules are often more sensitive to protease degradation. We therefore studied whether the ABCB1-I541F was more susceptible to *in situ* proteolysis, using isolated microsomes from MDCK cells stably transfected with either GFP-ABCB1-WT or GFP-ABCB1-I541F. The protocol was established previously for the ΔF508 cystic fibrosis transmembrane regulator (CFTR) mutant (17). Digestion with thermolysin or kallikrein revealed that compared with the wild-type protein, the mutant was sensitive to lower enzyme concentrations (Fig. 1). The majority of generated fragments were identical except for the largest peptides, which were underrepresented after digestion of the mutant. Because GFP is located at the C terminus of ABCB1, this finding suggested that the N-terminal moiety of the mutant was more sensitive to proteolysis. The fragment migrating at ~27 kDa, which appears to be rather resistant to both enzymes, presumably corresponds to GFP. An ~40-kDa fragment was resistant to kallikrein both in ABCB1 and ABCB1-I541F, again indicating that the C terminus of the molecule was less affected by the mutation. These proteolysis experiments provided evidence for a conformational defect of the I541F mutant.

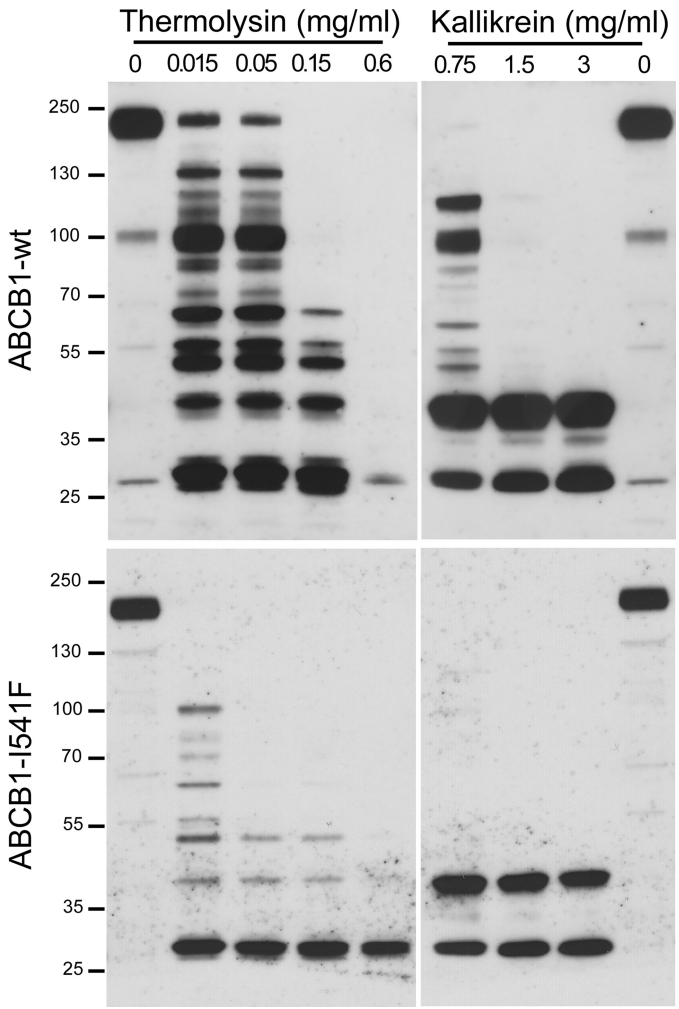

FIGURE 1. ***In situ* protease susceptibility of ABCB1-WT and ABCB1-I541F.** Microsomes obtained from MDCK cells expressing GFP-tagged ABCB1-WT or ABCB1-I541F were submitted to limited proteolysis at the indicated concentrations of thermolysin or kallikrein for 30 min at 4 °C. Samples (ABCB1, 30 μg and ABCB1-I541F, 50 μg protein per lane) were immunoblotted with the anti-GFP monoclonal antibody. Molecular mass standards are indicated in kDa.

The I541F Mutant Colocalizes and Coprecipitates with Calnexin—Calnexin is a chaperone of the ER that is involved in the folding of ABCB1 (19). To study whether the mutant interacts with calnexin, we performed colocalization and coimmunoprecipitation experiments using MDCK cells stably transfected with ABCB1-WT or ABCB1-I541F. By immunofluorescence, ABCB1-WT was detected at the cell surface, with only little colocalization with calnexin (Fig. 2*A*). By contrast, the ABCB1-I541F mutant colocalized strongly with calnexin, which appeared to be relocalized near the nucleus, together with the mutant (Fig. 2*A*). The change in calnexin pattern was probably due to accumulation of the mutant at

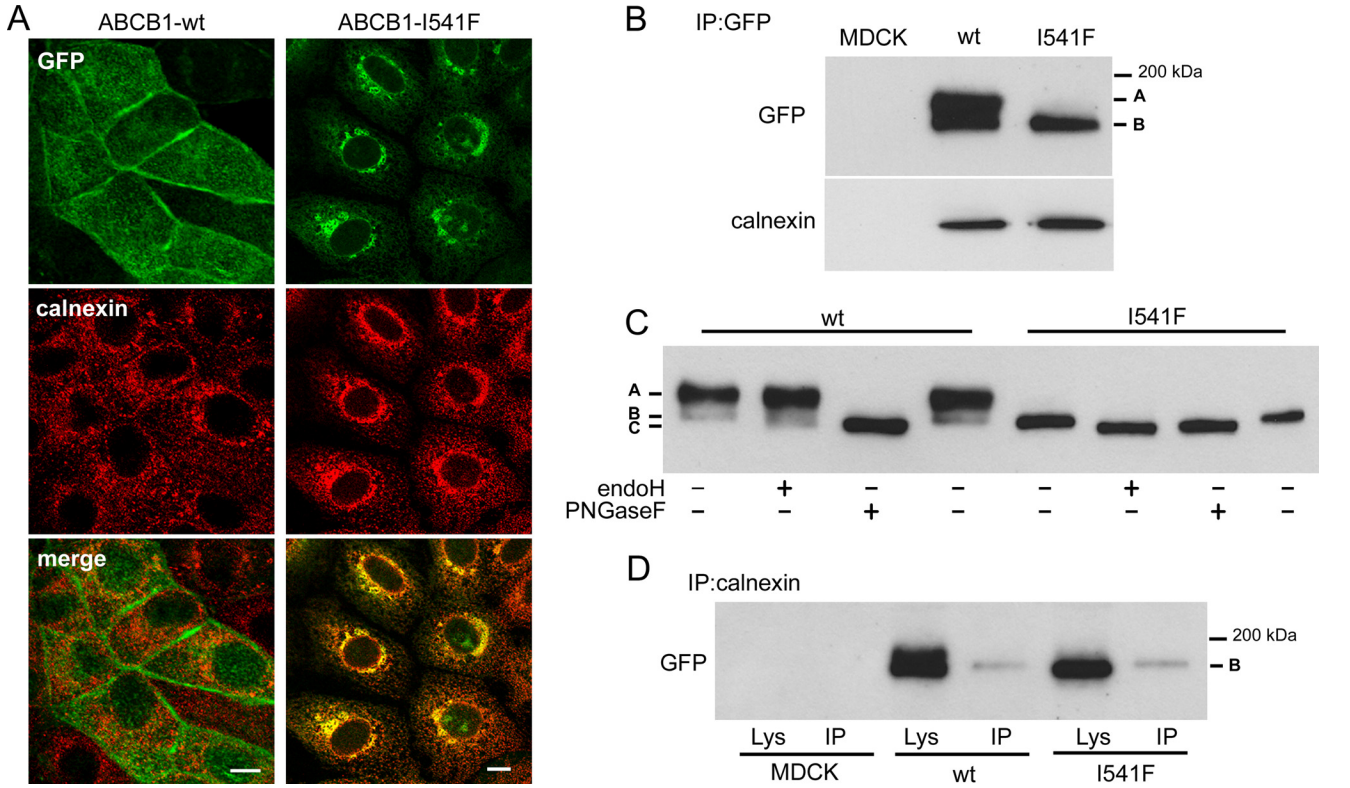

FIGURE 2. **ABCB1-I541F colocalizes and coprecipitates with calnexin.** *A*, MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F were fixed, and immunolocalization of calnexin was performed using a Cy3-conjugated secondary antibody. Confocal images show strong colocalization of the mutant with calnexin. Note that the staining pattern of calnexin in ABCB1-I541F cells is different from that in ABCB1-WT cells because of ER accumulation of the mutant. *Scale bars* = 10 μm. *B*, MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F and control MDCK cells were lysed, and immunoprecipitation (*IP*) was performed with the anti-GFP antibody. Samples were analyzed by immunoblotting using anti-GFP or anti-calnexin monoclonal antibodies. *C*, cell lysates were subjected to endoH or peptide *N*-glycosidase F digestion and processed for immunoblotting using the anti-GFP antibody. The mature (*A*), immature (*B*) and deglycosylated (*C*) forms are indicated. *D*, MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F and control MDCK cells were lysed, and immunoprecipitation was performed with the anti-calnexin antibody. Samples were analyzed by immunoblotting using the anti-GFP antibody. *Lys*, whole cell lysates.

specific sites of the ER. Immunoprecipitation experiments showed that calnexin coimmunoprecipitated with both ABCB1-WT and ABCB1-I541F (Fig. 2*B*). ABCB1 migrated as two bands of ~190 kDa and 175 kDa (bands A and B), whereas ABCB1-I541F presented only the 175-kDa band. Enzymatic deglycosylation of the samples by endoH or peptide *N*-glycosidase F showed that band A was not sensitive to endoH and thus corresponded to the mature glycosylated form, whereas band B, which was endoH-sensitive, corresponded to the immature high-mannose form (Fig. 2*C*). Immunoprecipitation of calnexin also pulled down ABCB1-WT and ABCB1-I541F. However, in this case, only band B, corresponding to the high mannose-form, was detected (Fig. 2*D*).

Effect of Calnexin Down-expression—Interaction between ABCB1-I541F and calnexin suggested that the chaperone might retain the I541F mutant in the ER. To test this hypothesis, we studied the fate of the mutant under calnexin depletion. Transfection of the cells with calnexin siRNA for 72 h allowed to substantially decrease the amount of calnexin (Fig. 3). This treatment, to a certain extent, increased the relative amount of immature ABCB1-WT, suggesting that calnexin is necessary for the processing of ABCB1-WT. However, it had little or no effect on the expression of ABCB1-I541F (Fig. 3) and did not induce the appearance of the mature form, which would have indicated a rescue effect.

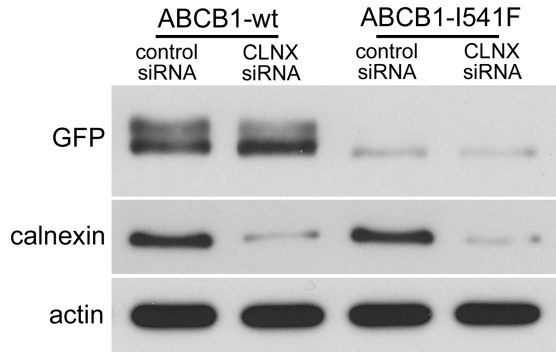

FIGURE 3. **Effect of calnexin silencing on ABCB1-I541F expression.** MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F were transfected with control or calnexin (*CLNX*) siRNA. After 72 h, cells were lysed, and samples were analyzed by immunoblotting using anti-GFP or anti-calnexin antibodies. Actin was taken as an internal standard. Shown is one representative of three experiments.

The I541F Mutant Coprecipitation with Hsc70 and Effect of Hsp70 Overexpression—The cognate Hsp70 family member Hsc70 is another ubiquitous chaperone that is involved in ABCB1 folding (20). We therefore tested whether the mutant was associated with Hsc70. By immunofluorescence, colocalization was very limited and restricted to discrete regions of the ER (Fig. 4*A*). However, Hsc70 coprecipitated significantly with the mutant ABCB1-I541F, and in higher amounts than with

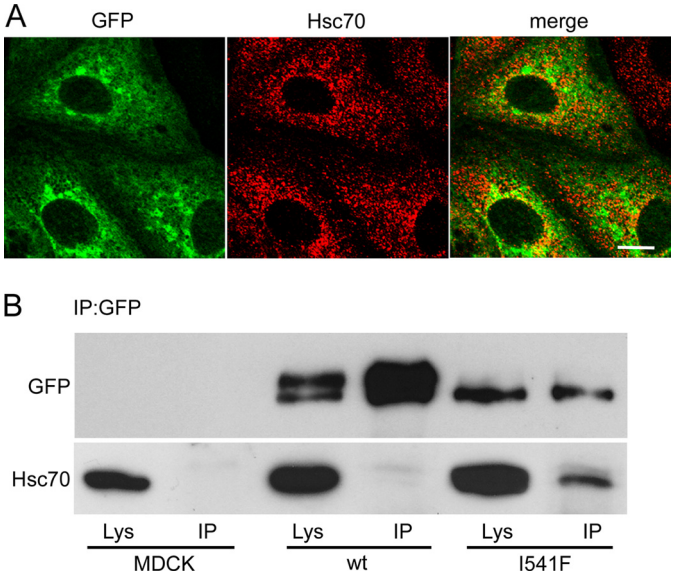

FIGURE 4. **ABCB1-I541F coprecipitates with Hsc70.** *A*, MDCK cells stably transfected with GFP-tagged ABCB1-I541F were fixed, and Hsc70 was detected by immunofluorescence using a Cy3-conjugated secondary antibody. Images were obtained by confocal microscopy. *Scale bar* = 10 μm. *B*, MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F and control MDCK cells were lysed, and immunoprecipitation (*IP*) was performed using the monoclonal anti-GFP antibody. Samples were analyzed by immunoblotting using anti-GFP or anti-Hsc70 monoclonal antibodies. Shown is one representative of three experiments. *Lys*, whole cell lysates.

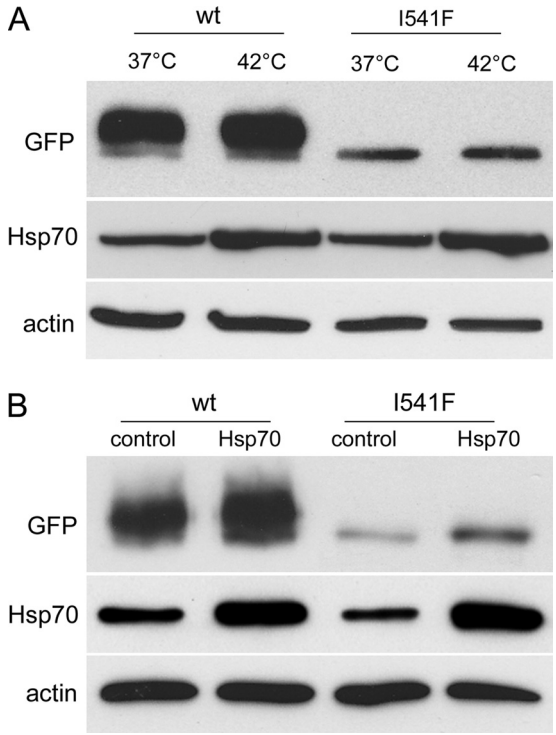

FIGURE 5. **Effect of Hsp70 overexpression on ABCB1-I541F expression.** *A*, MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F were maintained at 37 °C or exposed at 42 °C for 2 h and then returned to 37 °C for 24 h. Cells were lysed, and samples were analyzed by immunoblotting using anti-GFP and anti-Hsp70 antibodies. Shown is one representative of three experiments. *B*, MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F were transfected with the plasmid encoding Hsp70. After 48 h, cells were analyzed by immunoblotting using anti-GFP and anti-Hsp70 antibodies. Actin was taken as an internal standard. Shown is one representative of four experiments.

ABCB1-WT, indicating that this chaperone interacts preferentially with the mutant (Fig. 4*B*).

One of the functions of Hsc70 is to prevent aggregation of hydrophobic residues in the cytoplasm and to create best conditions for protein folding. Therefore, one can expect that increasing the amount of this chaperone might rescue folding, at least partially. Hsc70 is constitutively expressed, but its homolog Hsp70 can be induced in several stress conditions, especially by heat shock treatment (21). The cells were exposed for 2 h at the temperature of 42 °C and allowed to recover for 24 h at 37 °C. This treatment increased Hsp70 expression by ~2-fold (Fig. 5*A*). However, expression and maturation of the mutant did not change. We also increased Hsp70 expression by transient transfection of the cells with a cDNA encoding Hsp70. Hsp70 expression was increased at least 3-fold 2 days after transfection (Fig. 5*B*). In these conditions, the expression of ABCB1-I541F was notably increased but remained immature, indicating that it did not exit the ER (Fig. 5*B*).

Effect of Chemical Chaperones on ABCB1-I541F Expression—Different compounds referred to as chemical chaperones have been shown to rescue folding of mutant proteins. Among them glycerol, thapsigargin, and sodium 4-phenyl-butyrate (4-PB) were able to rescue cell surface expression of several membrane proteins, including the ΔF508 CFTR mutant (22, 23), the bile salt export pump (24), the K^+ channel (25), the low-density lipoprotein receptor (26), and the bone morphogenic receptor (27). ABCB1-I541F expressing cells were treated with thapsigargin or 4-PB at concentrations up to 100 μM for 24 h. The Western blot analysis pattern of ABCB1-I541F did not change in treated cells (Fig. 6*A*). Increasing the time of treatment did not allow the mutant to mature (not shown). Accordingly, the mutant was still located intracellularly (Fig. 6*B*). On the other hand, treatment with 7.5% glycerol increased the mature form of ABCB1-I541F on Western blot analyses (Fig. 6*C*). Increasing the concentration to 10% was toxic to the cells. Morphological studies showed that maturation was accompanied by some labeling of the apical cell surface (Fig. 6*D*). However, the mutant was still largely intracellular. Colocalization with cytoplasmic markers showed that after treatment with glycerol, the mutant best colocalized with GM130, a medial Golgi marker (Fig. 6*E*). This observation indicates that glycerol allows the mutant to exit the ER and improves its appearance at the cell surface, although its traffic may be slow.

ABCB1-I541F Is Functionally Rescued by Cyclosporin A—Cyclosporin A is an immunosuppressant known to be a substrate for and a competitive inhibitor of ABCB1 (28). It has been shown that several ABCB1 mutants could be rescued by treatment with cyclosporin A (29). We therefore tested the effect of cyclosporin A on the I541F mutant at concentrations ranging from 0. 1 to 10 μM. Cyclosporin A given for 24 h caused a dose-dependent increase in the mature form of ABCB1-I541F (Fig. 7*A* and *B*). On the other hand, cyclosporin A had no significant effect on ABCB1-WT (Fig. 7, *A* and *B*). Study of the localization of the rescued mutant by confocal microscopy showed that after cyclosporin A treatment, the I541F mutant was expressed at the apical membrane like the wild-type protein (Fig. 7*C*).

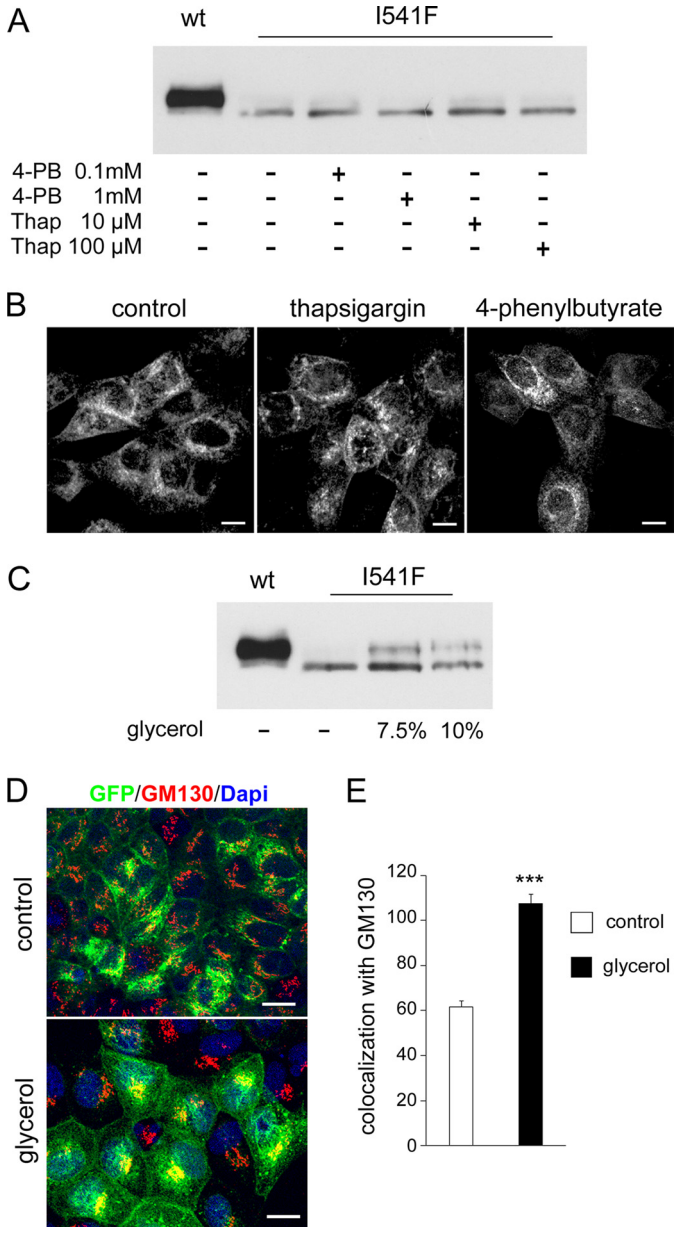

FIGURE 6. **Effect of chemical chaperones on the expression pattern of ABCB1-I541F.** *A*, MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F were treated with vehicle (-), 4-PB, or thapsigargin (*Thap*) at the indicated concentrations for 24 h. Immunoblotting was performed with anti-GFP. *B*, filter-grown MDCK cells stably transfected with GFP-tagged ABCB1-I541F were treated with vehicle (*control*), 100 μM thapsigargin, or 1 mM 4-phenylbutyrate for 24 h. Cells were fixed, and GFP fluorescence was analyzed by confocal microscopy. *Scale bars* = 10 μm. *C*, the same experiment as in *A*, except that cells were treated with 7.5% or 10% glycerol (w/v). *D*, MDCK cells stably transfected with GFP-tagged ABCB1-I541F were treated with 7.5% glycerol for 24 h. Cells were fixed and stained with the anti-GM130 antibody and a Cy3-conjugated secondary antibody. Nuclei were stained with DAPI. Images were obtained by confocal microscopy. *Scale bar* = 10 μm. *E*, the colocalization of GFP-tagged ABCB1-I541F with GM130 was quantified using the ImageJ 1.41 measure colocalization function on multiple confocal sections of at least 30 cells in three independent experiments. *Open bar*, control cells; *black bar*, glycerol-treated cells. Data (arbitrary units) are expressed as mean ± S.E. ***, $p < 0.001$.

To check whether the rescued mutant was functional, we used the calcein assay, which measures the capacity of cells to extrude calcein, a substrate for ABCB1. Calcein readily enters the cells and is converted to a fluorescent molecule in the cytoplasm. Accumulation of fluorescent calcein was measured in the presence or absence of the ABCB1 inhibitor verapamil in cells treated with different concentrations of cyclosporin A for 24 h. Extruded calcein was calculated as the difference between accumulated calcein in the presence and absence of verapamil. The assay could not be performed on cells treated with 5 or 10 μM of cyclosporin A because the combination of cyclosporin A at these concentrations with verapamil was toxic to the cells (data not shown). Fig. 7*D* shows that ABCB1-I541F MDCK cells treated with cyclosporin A extruded more fluorescent calcein than control cells in a dose-dependent manner. The difference was significant at the dose of 2 μM. These results show that the mutant rescued by cyclosporin was functional.

ABCB4-I541F Is Also Rescued by Cyclosporin A—Because cyclosporin A was very efficient at rescuing ABCB1-I541F, we also tested its potential effect on ABCB4-I541F. ABCB4-I541F was expressed at a low level in stably transfected MDCK cells and was not reproducibly detected by Western blotting. We therefore studied the effect of cyclosporin A in MDCK cells transiently transfected with the ABCB4-I541F plasmid. Six hours after transfection, the cells were treated with cyclosporin A, and the maturation of the mutant was examined 24 or 48 h later by immunoblotting. As shown previously (10), ABCB4-WT migrated as two bands corresponding to the immature and mature forms, whereas ABCB4 migrated essentially as the immature form (Fig. 8). A substantial amount of matured ABCB4-I541F was detected in cells treated with cyclosporin A at a concentration of 2 μM (Fig. 8, *A* and *B*). Increasing the concentration of cyclosporin above 2 μM did not increase the amount of the matured form. To confirm that a similar rescue would also occur in hepatic cells, $HepG_2$ cells were transfected with the ABCB4-I541F plasmid. Forty-eight hours later, cells were treated with 5 μM cyclosporin A. After 18 h of treatment, cells were fixed and processed for immunofluorescence. These cells are able to polarize and form bile canaliculi-like structures. In control cells, ABCB4-I541F was detected exclusively in the cytoplasm and was not detected at the membrane of bile canaliculi. After treatment with 5 μM cyclosporin A, the mutant was clearly detected at the membrane of bile canaliculi (Fig. 8*C*).

DISCUSSION

Single point mutations often produce molecules that are unable to fold correctly. These unfolded molecules may expose proteolytic cleavage sites and may be more susceptible to degradation. We found that the ABCB1-I541F mutant was more susceptible to the *in situ* action of proteolytic enzymes, especially in the first moiety of the molecule, where the mutation is located. This supported the assumption of a conformational defect that prevents the molecule to fold properly, and that makes it more sensitive to proteolysis. In keeping with this conclusion, it was shown previously that ABCB4 was undetectable by immunohistochemistry in the liver tissue from a patient bearing the ABCB4-I541F mutation (16). This suggests that in the liver *in vivo*, the improperly folded mutant protein is rapidly degraded.

Several strategies have been proposed to rescue the traffic of misfolded mutant proteins. A first approach is to manipulate

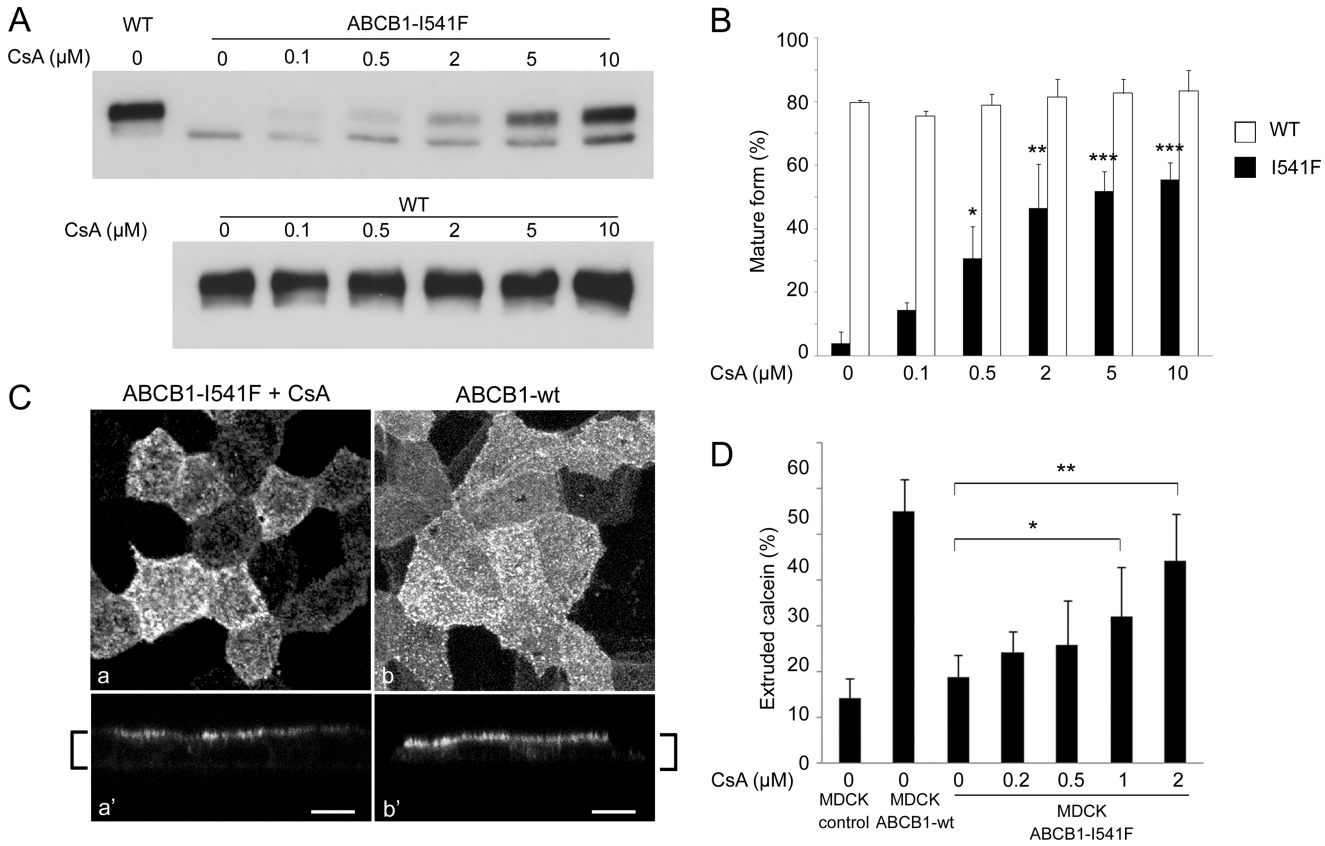

FIGURE 7. **Cyclosporin A rescues ABCB1-I541F.** MDCK cells stably transfected with GFP-tagged ABCB1-WT or ABCB1-I541F were treated with vehicle or cyclosporin A (*CsA*) at the indicated concentrations for 24 h. *A*, homogenates were analyzed by immunoblotting using the anti-GFP monoclonal antibody. *B*, quantification of experiments shown in *A*. The mature and immature bands were separately quantified on gels, and the relative amount of the mature form for each condition was calculated (mean ± S.D. of three experiments). *C*, confocal microscopy of MDCK cells stably transfected with GFP-tagged ABCB1-I541F and treated with 10 μM cyclosporin A (*a* and *a'*) and MDCK cells stably transfected with GFP-tagged ABCB1-WT (*b* and *b'*). *a* and *b* are *xy* projections. *xz* sections (*a'* and *b'*) show exclusive apical staining. *Brackets* indicate the height of the monolayer. *Scale bars* = 10 μm. *D*, control MDCK cells and MDCK cells stably transfected with GFP-tagged ABCB1-*wt*, or ABCB1-I541F were grown at 37 °C in 96-wells plates for 3 days. The cells were treated with the indicated concentrations of cyclosporin A 24 h before the calcein assay was performed. Results are expressed as the percentage of fluorescent calcein extruded after 60 min (mean ± S.D. of four determinations performed in triplicate). *, $p < 0.05$); **, $p < 0.01$; ***, $p < 0.001$.

endogenous cellular chaperones with the aim to facilitate the escape of mutant proteins from the quality control system and/or promote their folding. The cellular chaperone calnexin is one of the chaperones involved in quality control of the ER. Previous studies have shown that a number of mutant proteins exhibit prolonged interaction with calnexin (30, 31), including ABCB1 mutants (19). It has been suggested that calnexin binds the misfolded polypeptide and prevents its degradation (31). However, Okiyoneda *et al.* (32) found that the retention of ΔF508-CFTR in the ER was not rescued in calnexin KO cells. Similarly, we did not observe that calnexin silencing induced significant change in the expression of the ABCB1-I541F mutant and that it did not rescue ER retention. The inducible chaperone Hsp70 is another chaperone that has been targeted to improve the folding of mutant proteins. Induction of Hsp70 has been shown to promote ΔF508-CFTR maturation and its trafficking to the plasma membrane (33) and to rescue expression and function of a mutant cystathionine β-synthase (34). We found an increase in the ABCB1-I541F mutant when Hsp70 was overexpressed by transfection of the Hsp70 cDNA but not after heat shock treatment. The difference may be explained by the fact that transfection induced higher overexpression than heat shock. In both cases, no maturation of the mutant was observed.

A second approach to rescue the traffic of misfolded mutant proteins is to use chemical chaperones. Many of the chemical compounds that have been proposed are on the basis of the observation that function of the ER is intrinsically dependent on calcium concentrations. Thus, maintaining low calcium levels in the ER using the calcium pump inhibitor thapsigargin or other chemicals allows to correct abnormal protein trafficking of ΔF508 CFTR (23, 35) of certain mutants of the V2 vasopressin receptor (36) or of LQT2 channels (25). Another agent, 4-PB, is also able to functionally rescue a number of traffic-defective mutant membrane proteins, including ΔF508 CFTR (22, 33), mutants of the bone morphogenic protein receptor (27), of the epithelial sodium channel (37), the low-density lipoprotein receptor (26), ATP8B1 (38), and the ABC transporter ABCB11 responsible for biliary secretion of bile salts (24). The mechanism of 4-PB rescue is not yet well understood, but one of its effects is to reduce the expression of Hsc70 and increase Hsp70 (22, 39). Another class of chemical chaperones is represented by small osmolytes such as glycerol and organic solute that non-selectively stabilize mutant proteins and facilitate

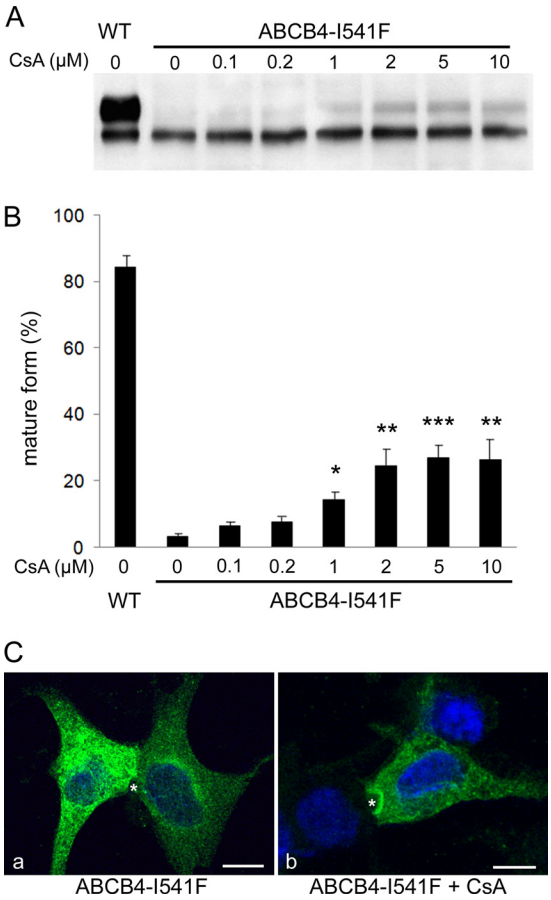

FIGURE 8. **Cyclosporin A rescues ABCB4-I541F.** *A*, MDCK cells were transfected with plasmids encoding ABCB4-WT or ABCB4-I541F. Six hours later, cyclosporin A (*CsA*) was added at the indicated concentrations. After 24 h, cells were lysed, and samples (40 μg protein) were analyzed by immunoblotting with an anti-ABCB4 antibody. *B*, quantification of experiments shown in *A*. The mature and immature bands were separately quantified on gels, and the relative amount of the mature form for each condition was calculated (mean ± S.D. of three experiments). *, $p < 0.05$); **, $p < 0.01$); ***, $p < 0.001$. *C*, $HepG_2$ cells transfected with the pCDNA3-ABCB4 plasmid were fixed, and ABCB4 was detected by immunofluorescence using an Alexa Fluor 488 secondary antibody. *a*, control cells; *b*, cells treated with 5 μM cyclosporin A for 18 h. Images are projections of focal sections obtained by confocal microscopy. Nuclei were stained with DAPI. The *asterisks* indicate the location of bile canaliculi. *Scale bars* = 10 μm.

their folding (40, 41). In our system, treatment with thapsigargin and 4-PB had no effect on the I541F mutant. Only glycerol did improve the maturation of the ABCB1-I541F mutant, which was able to exit the ER. However, the rescue was only partial, and the mutant remained still largely intracellular, although in a more distal compartment. It must be noted that the efficiency of chemical chaperones is very variable and appears to depend on each specific mutation. For instance, only six of 16 nephrin mutants were located at the cell surface after treatment with 4-PB (42). In the case of the vasopressin V2 receptor, only one of nine mutations showed improved maturation and plasma membrane rescue with glycerol and thapsigargin (36).

Finally, rescue of the I541F mutant was obtained with a specific substrate. Although chemical chaperones are not specific, pharmacological chaperones target one specific protein with which they interact. Loo and Clarke (43) showed that artificial mutations of ABCB1 that led to ER retention were corrected by a large variety of ABCB1 substrates. A similar strategy has been applied to improve folding and traffic of mutant membrane proteins responsible for genetic diseases using competitive inhibitors (44), antagonists (45, 46), or ligands (47). Here, we found that the ABCB1 inhibitor cyclosporin A was remarkably effective at restoring the traffic of ABCB1 bearing the I541F mutation. Cyclosporin A also rescued the ABCB4 mutant, albeit with somewhat less efficiency. This difference may reflect less affinity of ABCB4 than ABCB1 for cyclosporin A. The pharmacological chaperone may facilitate folding by constraining the protein to adopt a certain conformation (48). Therefore, efficient interaction between the chaperone and the protein being folded appears to be critical for rescue. The effect of cyclosporin A on ABCB4 maturation was moderate in MDCK cells, but a substantial amount of the mutant protein was detected at the surface of bile canaliculi in $HepG_2$ cells. We could not verify that the rescued mutant was functional in $HepG_2$ cells. However, the fact that the ABCB1 mutant was able to extrude calcein after cyclosporin A treatment suggests that rescued ABCB4-I541F may be functional as well. Although membrane expression of the ABCB4 mutant was not complete in our experimental conditions of cyclosporin A treatment, partial rescue in patients may be sufficient to avoid or at least postpone liver transplantation. These results open perspectives to treat PFIC3 by pharmacological means.

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